

# Dynamic modification of the ETS transcription factor PEA3 by sumoylation and p300-mediated acetylation

Baoqiang Guo, Niki Panagiotaki, Stacey Warwood and Andrew D. Sharrocks\*

Faculty of Life Sciences, University of Manchester, Michael Smith Building, Oxford Road, Manchester, M13 9PT, UK

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## ABSTRACT

Transcription factor activity is often controlled through the dynamic use of post-translational modifications. Two such modifications are acetylation and sumoylation, which both occur on lysine residues, providing the opportunity for cross-talk at the molecular level. Here, we focussed on the ETS-domain transcription factor PEA3 and studied the potential interplay between these two modifications. We demonstrate that PEA3 is acetylated in a p300-dependent manner. ERK MAPK pathway signalling potentiates acetylation of PEA3, and enhances its trans-activation capacity. However, the major acetylation and sumoylation events take place on the same sites in PEA3 making simultaneous modification impossible. Indeed, manipulation of either the sumoylation or acetylation pathways causes reciprocal changes in PEA3 acetylation and sumoylation respectively. However, despite the mutually exclusive nature of these modifications, both contribute to the trans-activation capacity of PEA3, implying that a dynamic series of modification events occurs during the activation process.

## INTRODUCTION

Transcription factor function is often controlled by post-translational modifications (1). Two such modifications are acetylation and sumoylation. As acetylation and sumoylation both occur on lysine residues, there is the possibility of direct functional antagonism between these post-translational modifications. Indeed there are several examples of where this direct antagonism through modification of the same lysine residue can occur and, in addition, where the two modifications can affect each other in a more indirect manner (reviewed in 2–6). However, in contrast to these antagonistic functions,

little is known about how acetylation and sumoylation might cooperate to either repress or activate transcription.

SUMO is a small polypeptide which is structurally related to ubiquitin, and is conjugated to substrates through a pathway composed of proteins, which show structural and functional homology with the ubiquitin pathway (reviewed in 7,8). Sumoylation is reversible, resulting in a dynamic process, which can be controlled in a number of ways such as through the action of protein kinase cascades (reviewed in 8,9). For example, HSF-1 sumoylation status is enhanced following heat shock-mediated phosphorylation of an extended SUMO recognition motif known as the phosphorylation-dependent sumoylation motif (PDSM) (10). This motif contains a proline-directed serine phosphorylation site located three amino acids downstream from the core consensus sumoylation motif ( $\psi$ KxE<sub>x</sub>SP). Sumoylation can cause changes in protein function in many different ways but in the case of transcription factors, it usually results in imparting repressive properties on transcriptional regulatory proteins (reviewed in 11,12). However, sumoylation can also result in transcriptional activation (reviewed in 13). Recently this was shown to be the case for the ETS-domain transcription factor PEA3 where sumoylation was not only required for efficient PEA3-mediated transcriptional activation but also for RNF4-directed ubiquitination and degradation of PEA3 (14).

PEA3 (also known as E1AF and ETV4) along with ER81/ETV1 and ERM/ETV5 are members of a subfamily of E-twenty six (ETS)-domain transcription factors. These three transcription factors share a common domain structure and exhibit extensive sequence similarity (reviewed in 15). Biologically, PEA3 subfamily members are often associated with cancer and in particular with the metastatic phase (reviewed in 15). During normal development, PEA3 has a role in neuronal pathfinding (16) and mammary gland development (17). PEA3 functions as a transcriptional activator protein and its trans-activation-capacity is enhanced following activation of the ERK and JNK MAP kinase signalling pathways (18).

\*To whom correspondence should be addressed. Tel: 0044 161 275 5979; Fax: 0044 161 275 5082; Email: a.d.sharrocks@manchester.ac.uk

Similarly, ER81 is regulated by the MAP kinase signalling cascades, which cooperate with the acetyl transferases PCAF and p300 to potentiate its trans-activation capacity (19). In the case of PEA3, sumoylation is enhanced by ERK MAP kinase signalling, and this contributes to the activating effect of this pathway (14).

A common unifying theme applying to PEA3 subfamily members appears to be the induction of a range of post-translational modifications such as acetylation and sumoylation by MAP kinase signalling. No links between these modifications within this subfamily have been studied. However, in other proteins such as HIC1, an extended core sumoylation motif ( $\psi$ KxEP) directs antagonistic acetylation and sumoylation of the embedded lysine residue (20). Here, we focussed on PEA3 and demonstrate that it is acetylated in a p300-dependent manner. This acetylation is potentiated by ERK MAPK pathway signalling, and enhances the trans-activation capacity of PEA3. Importantly, acetylation and sumoylation take place on the same sites in PEA3 making simultaneous modification impossible. Indeed, experimental manipulation of either the sumoylation or acetylation pathways causes reciprocal changes in PEA3 acetylation and sumoylation respectively. Despite the mutually exclusive nature of these modifications, both contribute to the trans-activation capacity of PEA3 and lie downstream of ERK pathway signalling, implying that a dynamic series of modification events occurs during the activation process.

## MATERIALS AND METHODS

### Plasmid constructs

The following plasmids were used in mammalian cell transfections. pCH110 (Pharmacia), p5xPEA3 RE-Luc (5× PEA3/ETS binding sites and AdML promoter; 21), pColI-Luc [containing the *MMP1* promoter (−517/+63); kindly provided by Olivier Kassel; (22)] and pHES2 (nucleotides −1432 to +59 of human *COX-2* promoter; 23) have been described previously. pAS1801 (encoding full-length mouse PEA3) (24), pKW2T-HA-PIASy (kindly provided by Frances Fuller-Pace), pCDNA3-Ubc9 and pCDNA3-His-SUMO-2 (kindly provided by Ron Hay), pCMV-p300 and pCMV-p300ΔHAT [kindly provided by Michael Brattain; (25)] and Flag-E1AF (encoding human PEA3) [kindly provided by Tamotsu Nishida; (26)] have been described previously. pCMV-MEK1 encodes constitutively active MEK-1 (ΔNS218E-S222D).

The following plasmids encoding full-length PEA3 with mutations in combinations of the lysine residues K96 (K1), K222 (K2), K256 (K3), K328 (K4), K437 (K5) or glutamate residues E98 (E1), E224 (E2), E258 (E3), E330 (E4), E439 (E5) were described previously (14). pAS1043, pAS1050 pAS1029, pAS1030, pAS1031, pAS1032 pAS1034 pAS2651 pAS1040 pAS1042 and pAS1041 [encode PEA3(E1A), PEA3(E12345A), PEA3(K1R), PEA3(K2R), PEA3(K3R), PEA3(K4R), PEA3(K12R), PEA3(K1345R), PEA3(K1234R), PEA3(K2345R) and PEA3(K12345R), respectively]. pAS1037, pAS1038,

pAS1039, pAS1040, pAS4051 and pAS4053 [encoding PEA3(K34R), PEA3(K134R), PEA3(K234R), PEA3(K1234R), PEA3(K1Q) and PEA3(K12Q), respectively] were constructed using the appropriate primer pairs (14), ADS4057/ADS4058 (K1Q), or ADS4059/ADS4060 (K2Q) on templates already containing individual or multiple mutations.

For bacterial expression, pGEX-KG-PEA3(1-335) (pAS2502) was constructed by inserting a BamHI/EcoRI-cut PCR product (generated using the primer pair ADS1580/ADS1582 on the template pAS1801) into the same sites in pGEX-KG.

### Tissue culture, cell transfections, reporter gene assays and RT-PCR

HEK293 and F9 cells were grown in DMEM supplemented with 10% foetal bovine serum. Where indicated, cells were treated with phorbol 12-myristate 13-acetate (PMA) (10 nM), the MEK inhibitor U0126 (10 μM), TSA (200 nM) or the proteasome inhibitor MG132 (5 μM). Plasmid transfections for HEK293T cells were performed using Polyfect (Qiagen) according to the manufacturer's instructions. SiRNA transfections were generally performed with 5 μl lipofectamine 2000 per well in a 6-well plate and 40 nM SMARTpool siRNA duplexes against PCAF and p300 or a scrambled duplex (Dharmacon). For p300 knockdown in *COX-2* promoter studies Lipofectamine RNAiMAX (Invitrogen) was used by adding 2 μl of p300 siRNA (10 μM) to 200 μl of Opti-MEM I medium without serum to a 12 well tissue culture plate, mixed gently with 2 μl of Lipofectamine RNAiMAX and left to incubate for 10–20 min at room temperature. Diluted 293 T cells were added to each well. After 24 h, pHES2 and PEA3 expression plasmids were transfected using Polyfect (Qiagen). After another 24 h, cells were harvested and used for luciferase assays.

For reporter gene assays, typically 0.25 μg of reporter plasmid and 50 ng of pCH110 were co-transfected with 0.005–2 μg of expression plasmids. Cell extracts were prepared and equal amounts of protein were used in luciferase and β-galactosidase assays as described previously (24). Real-time RT-PCR was performed as described previously using the primer pairs ADS1962/ADS1963 to detect *MMP-1* expression (14). *MMP-1* levels were normalized to 18S RNA levels.

### In vivo SUMO assays

*In vivo* sumoylation of overexpressed proteins was detected by co-transfection of His-tagged SUMO-2 and Ubc9 and wild-type (WT) or mutant PEA3 derivatives into 293T cells. Cell lysates were made under denaturing conditions in the presence of 8 M guanidine-HCl, and 10 mM imidazole and sumoylated conjugates isolated by Ni-affinity chromatography as described previously (27).

### In vitro acetylation assays

For *in vitro* acetylation assays, ~2 μg of GST-PEA3 fusion protein or histones were added to 20 μl reactions containing 250 mM Tris pH 8.0, 25% glycerol, 250 mM KCl, 0.5 mM EDTA, 1 mM DTT, 10 mM sodium butyrate,

0.05  $\mu\text{Ci}$  of [ $^{14}\text{C}$ ]acetyl-CoA (59 mCi/mmol) and  $\sim 100$  ng p300 (HAT domain) (Millipore). The samples were incubated at  $30^\circ\text{C}$  for 2 h then terminated by adding 5  $\mu\text{l}$  SDS loading buffer and boiling before subjecting to SDS-PAGE.  $^{14}\text{C}$ -labelled bands were visualized by phosphorimaging and analysed using Quantity One software (Biorad).

### Western blot and co-immunoprecipitation analysis

Western blotting was carried out with the primary antibodies; PEA3 (Santa Cruz, sc113), Flag (Sigma), anti-acetyl lysine (Ab409-200; Abcam), anti-HA (Cancer Research UK), GAPDH (Abcam) and p300 (Santa Cruz, N-15) essentially as described previously (24). Data were quantified using Quantity One software (Biorad).

Co-immunoprecipitation of overexpressed proteins was performed as described previously (24). To perform co-immunoprecipitations of endogenous proteins from F9 cells and to precipitate PEA3 for subsequent analysis of acetylation levels, we followed the method essentially as previously described (28). To enhance the stability of PEA3 in F9 cells, cells were pre-treated with MG132 for 6 h. Briefly, the cell pellets were resuspended in 2 pellet volumes of Buffer A (20 mM HEPES, pH 7.9, 10 mM NaCl, 3 mM  $\text{MgCl}_2$ , 0.1% NP-40, 10% glycerol, 0.2 mM EDTA, plus protease inhibitors) and left on ice for 10–15 min with occasional tapping. The nuclei were pelleted by spinning down at 2000 rpm for 5 min at  $4^\circ\text{C}$ . The nuclei were washed with 3 pellet volumes of Buffer B (20 mM HEPES, pH 7.9, 20% glycerol, 0.2 mM EDTA, protease inhibitors) and centrifuged again. The pelleted nuclei were then resuspended in one pellet volume of Buffer C (20 mM HEPES, pH 7.9, 400 mM NaCl, 20% glycerol, 0.2 mM EDTA, plus protease inhibitors) and agitated for 45 min at  $4^\circ\text{C}$ . After centrifugation for 15 min at 13000 rpm, the supernatants were diluted to one ml with washing buffer (pH 7.5, 20 mM Tris-HCl, 150 mM NaCl). PEA3 antibody was added, incubated overnight at  $4^\circ\text{C}$ . The following day Protein G beads (Sigma) were added and incubated for 2 h and then washed with washing buffer four times.

### Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) assays using anti-HA antibody were performed as described previously (14). Bound promoters were detected by PCR using primers for the promoter region (ADS1277/ADS1278) or coding (ADS1279/ADS1280) region of p5xPEA3 RE-Luc (24). ADS1968, 5'-GGGGACTCCAAGGCTCTATT-3' and ADS1969, 5'-TCAGGAAAGCAGCATGTGAC-3'.

### Sample preparation, mass spectrometry and data analysis

Four 10 cm dishes of 293 cells co-transfected with expression vectors for PEA3 and p300 were serum starved for 24 h, then stimulated with PMA 6 h. PEA3 was then immunoprecipitated, subjected to SDS-PAGE and the region of the gel corresponding to the location of the PEA3-specific band was excised. Samples were reduced, alkylated with iodoacetamide, and then digested overnight with trypsin, GluC or GluC and LysC. Digested samples

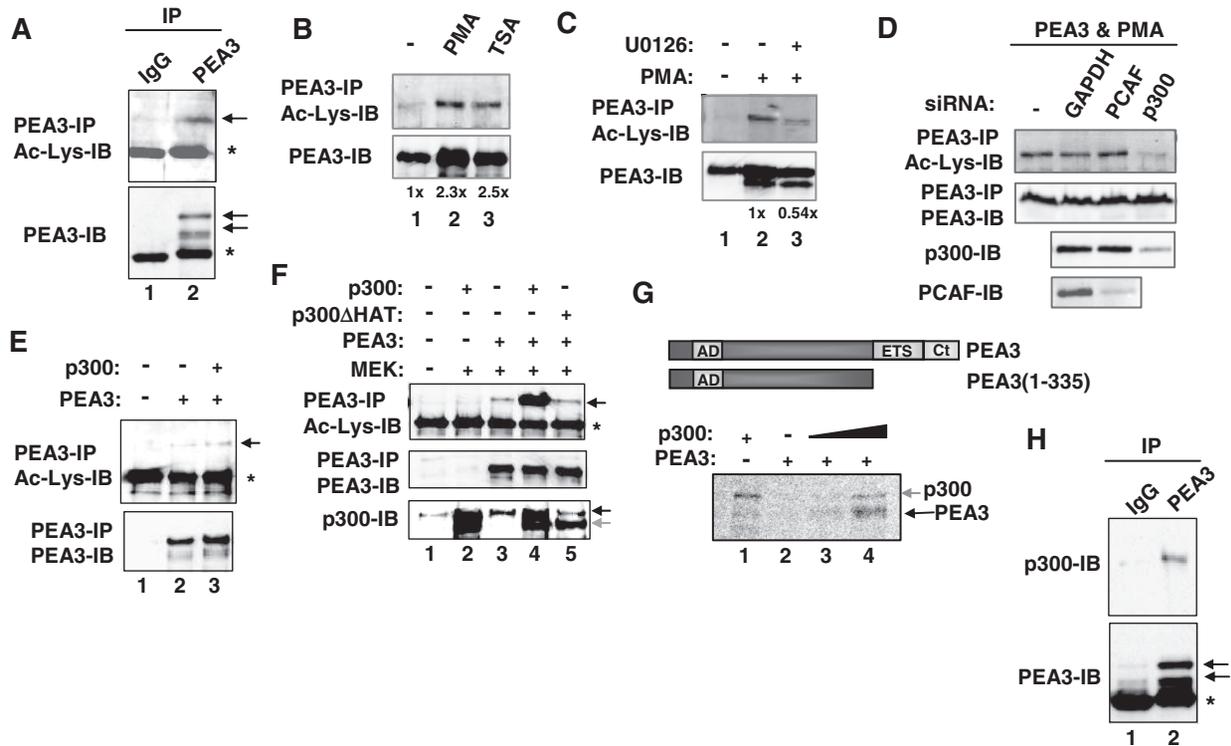
were analysed by LC-MS/MS using a NanoAcquity LC (Waters, Manchester, UK) coupled to a LTQ Velos (Thermo Fisher Scientific, Waltham, MA, USA). Peptides were concentrated on a pre-column (20 mm  $\times$  180  $\mu\text{m}$  i.d., Waters). The peptides were then separated using a gradient from 99% A (0.1% FA in water) and 1% B (0.1% FA in acetonitrile) to 30% B, in 40 min at 200  $\text{nl min}^{-1}$ , using a 75 mm  $\times$  250  $\mu\text{m}$  i.d. 1.7  $\mu\text{M}$  BEH C18, analytical column (Waters). Peptides were selected for fragmentation automatically by data-dependant analysis.

Data produced were searched using Mascot (v 2.2, Matrix Science, UK), against the IPI Mouse database with the appropriate enzyme selected, carbamidomethylation of cysteines and acetylation of lysines selected as fixed and variable modifications, respectively. Data were then validated using Scaffold (Version 3, Proteome Software, Portland, OR) and identified sites of modification confirmed by manual inspection of product ion spectra.

## RESULTS

### PEA3 is acetylated by p300

The PEA3 subfamily member ER81 has been shown to be acetylated by both p300 and PCAF (19). Here we tested whether PEA3 is also acetylated. Endogenous PEA3 is expressed in mouse embryonic carcinoma F9 cells (29) and was precipitated using anti-PEA3 antibody and acetylation detected using an anti-acetyl lysine antibody. Two bands corresponding to PEA3 were identified, and acetylation of the upper band was detected (Figure 1A). We also asked whether acetylation of exogenous PEA3 could also be detected by transfecting HEK293 cells with a mouse PEA3 expression vector. Weak acetylation was detected but this could be significantly enhanced through either treatment of the cells with either PMA or with the histone deacetylase (HDAC) inhibitor TSA (Figure 1B). TSA treatment alters the dynamics of acetylation by inhibiting the activity of Class I and II HDACs while PMA is known to enhance the trans-activation activity of PEA3 in an ERK MAP kinase pathway-dependent manner (14). The enhancement of PEA3 acetylation by PMA is also dependent on ERK pathway signalling as the MEK inhibitor U0126 reduces the amount of inducible PEA3 elicited by PMA treatment (Figure 1C). The identity of the acetyl transferase responsible for PEA3 acetylation was probed by siRNA-mediated depletion of PCAF or p300. These experiments were carried out in the presence of PMA to activate the ERK pathway. Depletion of p300 caused a substantial decrease in PEA3 acetylation levels whereas depletion of PCAF had little effect on PEA3 acetylation (Figure 1D), thereby specifically implicating p300 in PEA3 acetylation. This link was further investigated by overexpression of p300 which resulted in enhanced acetylation of PEA3 both in the absence of ERK pathway activation (Figure 1E, lane 3) or when this pathway is activated by co-transfection of constitutively active MEK (Figure 1F, lane 4). Importantly, the



**Figure 1.** PEA3 is acetylated by p300. (A) Endogenous PEA3 was immunoprecipitated IP from F9 cells with anti-PEA3 antibody or non-specific IgG and resulting bands were detected by immunoblotting (IB) using anti-PEA3 or anti-acetyl lysine (Ac-Lys) antibodies. (B–F) HEK293 cells were transfected with a PEA3 expression vector, followed by IP with anti-PEA3 antibody and IB with either anti-PEA3, acetyl lysine, or p300 antibodies. Cells were treated with (B) PMA or TSA for 6h, (C) PMA for 6h, (D) siRNAs against p300 or PCAF, (E and F) cotransfection with p300 and p300 $\Delta$ HAT expression vectors. Where indicated, cells were treated with PMA (D) or a vector encoding constitutively activated MEK was co-transfected (F). Bands corresponding to full-length (black arrow) and  $\Delta$ HAT (grey arrow) versions of p300 are indicated in (F). Quantification of the level of PEA3 acetylation (normalized for total PEA3 levels) is shown below the figures relative to the amount in the absence of treatment (B) or the presence of PMA (C) (taken as 1). (G) *In vitro* acetylation assays of GST-PEA3(1-335) with recombinant p300 catalytic domain. A phosphorimage showing  $^{14}$ C-labelled acetylated species is shown. Arrows indicate the location of bands corresponding to p300 autoacetylation (grey) and acetylated PEA3 (black). The schematic diagram shows the fragment of PEA3 used here (1-335) compared to full-length PEA3. (H) Co-immunoprecipitation analysis of endogenous PEA3 with endogenous p300 from F9 cells. IP was performed with non-specific IgG or an anti-PEA3 antibody and IB with either anti-PEA3, or p300 antibodies. Bands corresponding to PEA3 are indicated by arrows. Asterisks indicate bands arising from cross-reactivity with the antibody heavy chain.

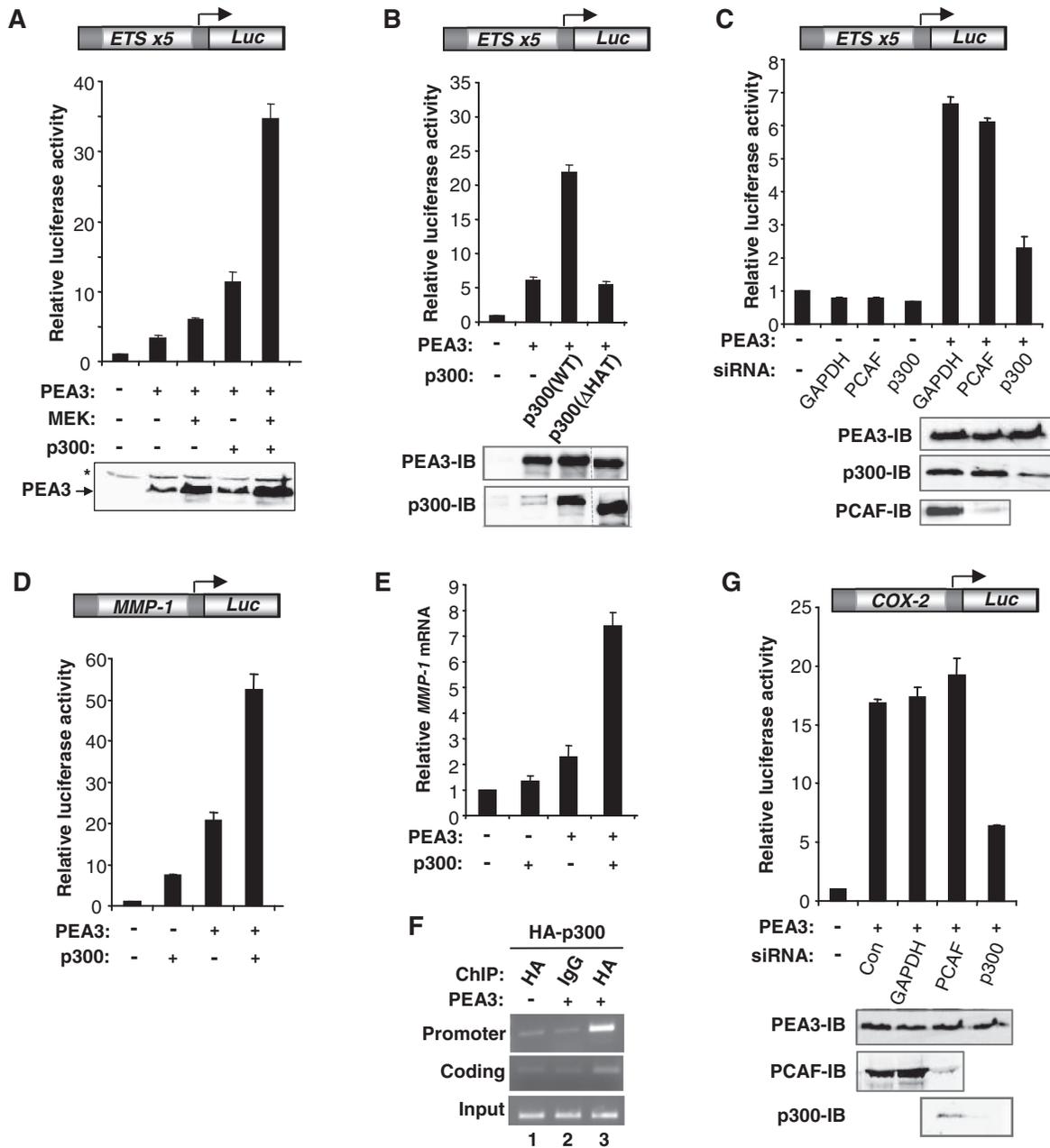
catalytic activity of p300 is required, as a mutant version lacking the histone acetyltransferase domain ( $\Delta$ HAT) is unable to potentiate PEA3 acetylation (Figure 1F, lane 5). To determine whether p300 can directly acetylate PEA3, we tested the ability of recombinant p300 HAT domain to acetylate a purified truncated version of PEA3 [GST-PEA3(1-335)] which contains the N-terminal part of PEA3 that precedes the ETS DNA binding domain. Acetylation of PEA3 was detectable in this assay (Figure 1G). Finally we asked whether p300 could bind to PEA3. Endogenous PEA3 was precipitated from F9 cells and p300 was found to co-precipitate with PEA3 (Figure 1H).

Together these results demonstrate that PEA3 is acetylated in a p300-dependent manner and this acetylation is enhanced following ERK pathway activation.

### p300 promotes transcriptional activation by PEA3

To study transcriptional activation by PEA3 in the absence of other promoter-bound transcription factors, we used a luciferase reporter construct driven by a basal promoter and five reiterated ETS binding motifs. PEA3 is

sufficient to enhance the activity of this reporter construct but this activity is substantially increased by either co-expression of a constitutively active form of MEK to activate the ERK pathway or the acetyl transferase p300 (Figure 2A; Supplementary Figure S1). Co-transfection of expression vectors encoding PEA3, MEK and p300 further increased the reporter activity in a synergistic manner (Figure 2A). To probe whether the acetyltransferase activity of p300 was required for enhancing the trans-activation capacity of PEA3, we compared the effect of WT p300 and p300 $\Delta$ HAT which lacks its HAT domain. While WT p300 potentiated PEA3-mediated trans-activation, no such increase was observed with p300 $\Delta$ HAT (Figure 2B). Conversely, we removed endogenous p300 through siRNA-mediated depletion. The loss of p300 ablated PEA3-mediated transcriptional activation whereas PCAF depletion had little effect (Figure 2C). Finally, we asked whether p300 can also affect the activity of PEA3 on one of its target genes, *MMP-1*. p300 was able to promote PEA3-mediated activation of both a luciferase reporter driven by the *MMP-1* promoter (Figure 2D) and PEA3-mediated expression of



**Figure 2.** p300 potentiates the trans-activation capacity of PEA3. (A–C) Reporter gene analysis using a luciferase reporter construct driven by five PEA3 (ETS) binding sites (shown schematically at the top). HEK293 cells were transfected with PEA3 where indicated and co-transfected with combinations of (A) MEK and p300, (B) p300 or p300ΔHAT (0.5 μg) or (C) control siRNA duplexes against GAPDH or siRNA duplexes against p300 or PCAF. Western blots show the levels of PEA3 and p300 under these conditions (dashed lines illustrate where two parts of a gel have been rejoined). (D) Reporter gene analysis using a luciferase reporter construct driven by the *MMP-1* promoter (shown schematically at the top). HEK293 cells were transfected with vectors encoding PEA3 and p300 where indicated. Luciferase activities are the average of duplicate samples and are representative of two to three independent experiments. Activities are shown relative to the activity of the reporter alone (taken as 1). (E) RT-PCR analysis of endogenous *MMP-1* expression in HEK293 cells in the presence of the indicated combinations of co-transfected expression vectors for PEA3 and p300. Data are the average of duplicate samples. (F) ChIP assays with anti-HA antibody, or control IgG in HEK293 cells co-transfected with the ETSx5-luciferase reporter plasmid, PEA3 (where indicated) and HA-tagged p300 expression constructs where indicated. The signals on the promoter region and on the coding region of the luciferase reporter plasmid are shown. (G) Reporter gene analysis using a luciferase reporter construct driven by the *COX-2* promoter (shown schematically at the top). HEK293 cells were co-transfected with a vector encoding PEA3 and a series of siRNA duplexes where indicated.

the endogenous *MMP-1* gene (Figure 2E). We also performed ChIP analysis of HA-tagged p300 binding to the luciferase reporter construct to probe the role of PEA3 in p300 recruitment. p300 binding to the promoter region

was strongly enhanced upon co-transfection of PEA3 (Figure 2F, lane 3) but little binding was visible when non-specific IgG was used (Figure 2F, lane 2) or binding to the luciferase coding region was analysed (Figure 2F,

middle panel). Furthermore, p300 potentiated the activity of PEA3 on another target gene *COX-2* (data not shown) and, conversely, depletion of p300 reduced the ability of PEA3 to activate the *COX-2* promoter (Figure 2G).

Collectively, this combination of overexpression and knockdown experiments demonstrate that p300 plays an important role in PEA3-mediated transcriptional activation.

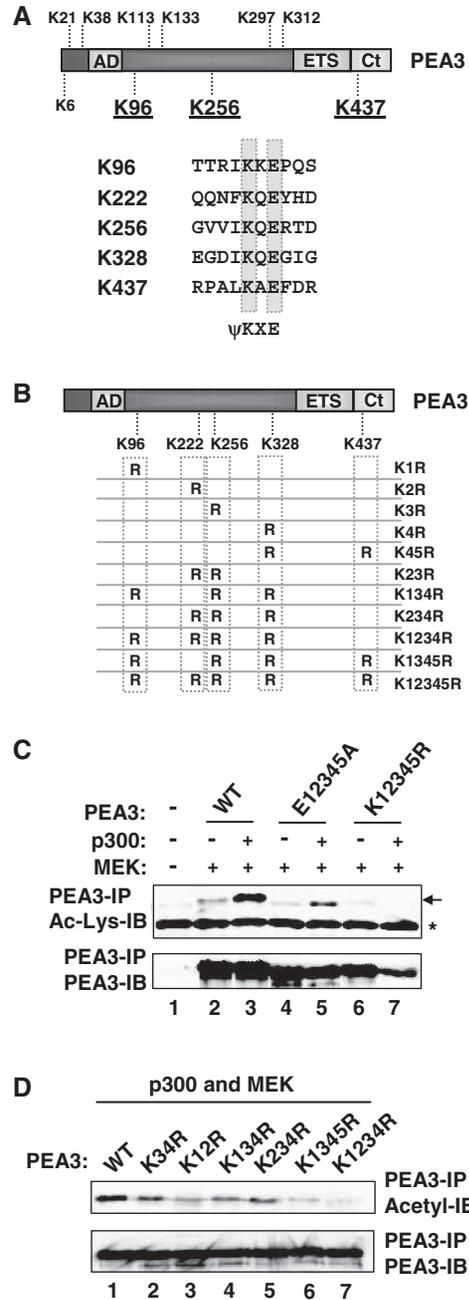
### Mapping the p300-mediated acetylation sites in PEA3

To further study the functional importance of p300-mediated acetylation events, we mapped the acetylation sites in PEA3. HEK293 cells were co-transfected with PEA3 and p300, extracts were separated by SDS-PAGE and regions corresponding to the predicted location of PEA3 were excised, subjected to proteolytic digestion and analysed by mass spectrometry. Ten different sites of acetylation could be identified (Figure 3A). Interestingly, three of these are on lysines (K96, K256 and K437) which conform to the core consensus sumoylation site  $\psi$ KxE (Figure 3A), and two of these, K96 and K256 were recently shown to be modified by SUMO *in vivo* (14,26,30). This suggested a possible interplay between these two modifications, so we focussed on the five lysine residues embedded within core SUMO motifs, and mutated these individually and in combination (Figure 3B). WT and mutant PEA3 proteins were then tested for p300-mediated acetylation *in vivo*. Mutation of all five lysine residues resulted in virtually a complete loss of PEA3 acetylation (Figure 3C, lanes 6 and 7). However, in comparison, mutation of the five glutamic acid residues in the core sumoylation motif caused a much less dramatic change in acetylation levels (Figure 3C, lanes 4 and 5). This mutant exhibits substantially reduced sumoylation levels (14), suggesting that efficient sumoylation is not a prerequisite for subsequent PEA3 acetylation. Further combinations of four lysine mutations also led to substantial decreases in acetylation, whereas triple and double mutations had variable effects with mutation of the first two lysine residues having the biggest effect (Figure 3D).

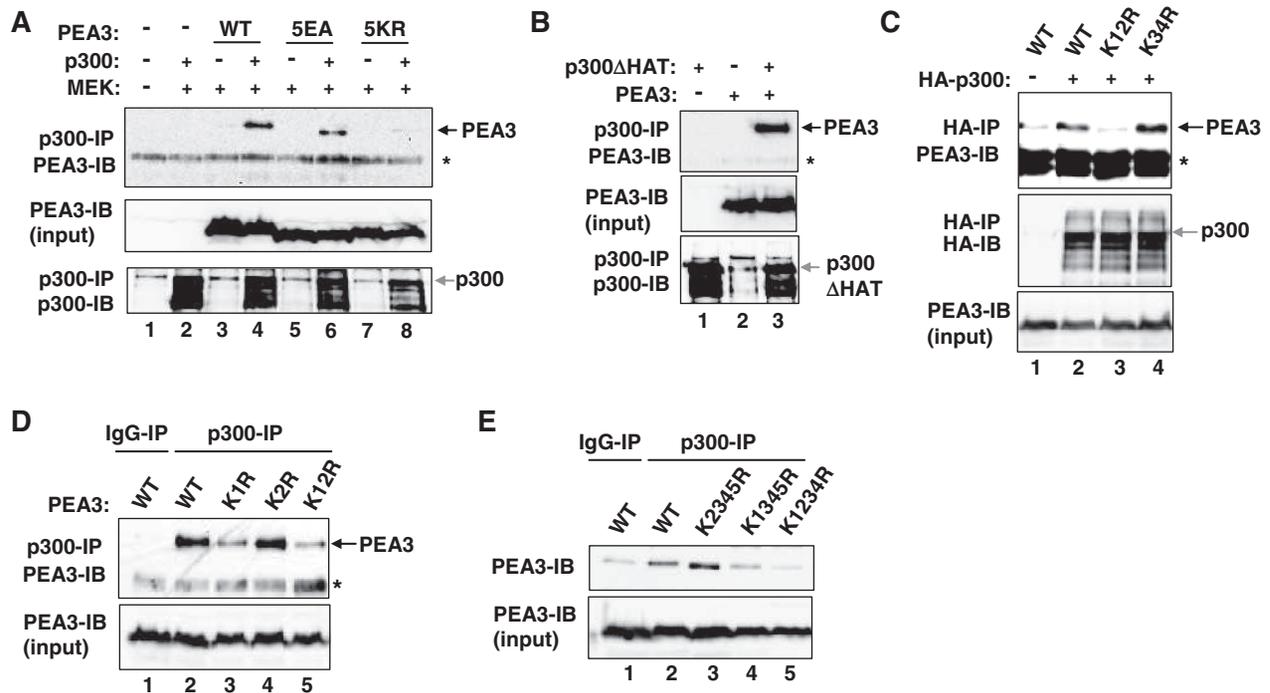
These results therefore demonstrate that p300-mediated acetylation of PEA3 takes place at a set of sites that overlap with sites that are also subject to sumoylation. Multiple sites appear to be important for acetylation with K96 and K222 playing the most prominent roles.

### Mapping the p300 binding determinants in PEA3

Next we wished to determine whether the lysine residues in PEA3 played a role in p300 binding. Again we focussed on the lysine residues embedded in the core sumoylation motifs. Mutation of all five lysine residues in PEA3 resulted in virtually a complete loss of p300 binding (Figure 4A, lanes 7 and 8). However, mutation of the five glutamic acid residues in the core sumoylation motif only slightly reduced p300 binding (Figure 4A, lanes 5 and 6). The acetyltransferase activity of p300 was unimportant for this binding as p300 $\Delta$ HAT bound efficiently to PEA3 (Figure 4B). Next, we further delineated the lysine residues required for p300 binding through analysing a series of double and single mutant proteins. The first two lysine



**Figure 3.** Mapping the acetylation sites in PEA3. (A) Schematic showing the locations of the acetylation sites detected by mass spectrometry in PEA3 precipitated from HEK293 cells co-transfected with PEA3 and p300 expression vectors and treated with PMA for 6 h. Underlined residues represent lysines which reside in consensus core sumoylation motifs. The sequences surrounding lysine residues in PEA3 conforming to the core  $\psi$ KxE consensus sequence for sumoylation are shown at the bottom. (B) Schematic representation of the locations of the lysine residues in PEA3 subjected to the indicated arginine mutations. The numbering on the right is a simplified nomenclature used in subsequent figures. (C and D) IP-western analysis of PEA3 acetylation. PEA3 was IP from HEK293 cells and acetylation detected by IB with anti-acetyl lysine antibody. Cells were co-transfected with expression vectors encoding the indicated WT and mutant PEA3 proteins, the indicated combinations of MEK and p300 expression vectors. The asterisk indicates bands arising from cross-reactivity with the antibody heavy chain.



**Figure 4.** Role of lysine residues in p300 binding. (A–E) Co-immunoprecipitation analysis of PEA3 with p300 and PCAF. p300 was IP from HEK293 cells co-transfected with expression vectors encoding the indicated combinations of p300 or p300 $\Delta$ HAT with WT and mutant PEA3 proteins, and co-precipitated PEA3 was identified by IB. Where indicated, non-specific IgG was used as a control in the IP. Asterisks indicate bands arising from cross-reactivity with the antibody heavy chain whereas arrows indicate bands corresponding to PEA3 (black) or p300 (grey).

residues were identified as critical for efficient p300 binding, whereas the third and fourth lysines played lesser roles (Figure 4C). Of these first two residues, the first, K96, was the most critical for p300 binding as mutation of this residue on its own substantially reduced p300 binding (Figure 4D). K96 in PEA3 is therefore important specifically for p300 binding. To test whether this residue is sufficient for p300 binding, we mutated all the other lysine residues embedded within SUMO consensus motifs and tested for p300 binding. This mutant, PEA3(K2345R) was still able to efficiently bind to p300, in contrast to other mutants retaining either the second or fifth lysine residues, which exhibited much reduced binding to p300 (Figure 4E).

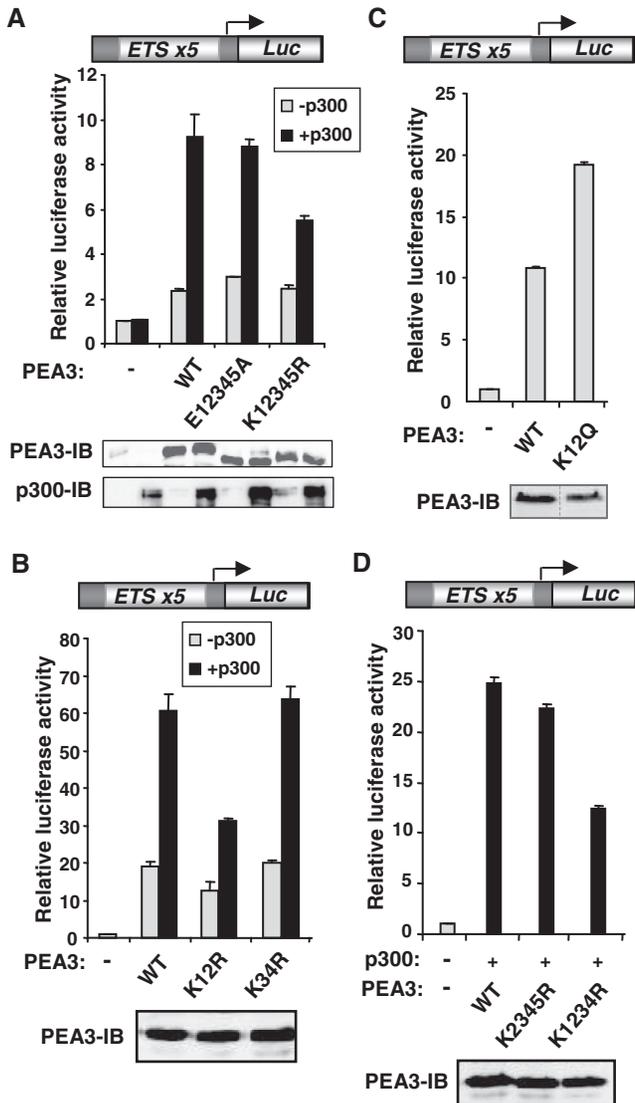
Together, these results demonstrate an important role for K96 in PEA3 for binding to p300, and further demonstrate that the requirements for p300 binding closely mirror the requirements for p300-mediated acetylation.

#### The role of p300 acetylation sites in PEA3-mediated transcriptional activation

To probe the role of the acetylation sites in PEA3-mediated trans-activation, we used a reporter assay system to assess the ability of p300 to potentiate the activity of a panel of PEA3 mutants in the context of reiterated PEA3 binding elements. The mutation of all five lysine residues embedded in sumoylation motifs caused a reduction in p300-mediated increases in PEA3 activity (Figure 5A). However, the equivalent mutant which destroys the core SUMO motifs by removing the glutamic acid residues, had little effect on p300-mediated

trans-activation levels (Figure 5A), demonstrating that the loss of sumoylation was not the reason for the reduced activity. Further mutations of individual lysine residues had variable effects on p300-mediated potentiation of PEA3 activity (Supplementary Figure S2A) whereas compound sets of four mutations severely affected the activity of p300 towards PEA3 in this context (Supplementary Figure S2B). The combination of mutating K96 and K222 in the PEA3(K12R) mutant had the biggest effect on reducing PEA3 acetylation (Figure 3) and p300 binding (Figure 4) and individual mutations at these sites had a moderate effect on PEA3 activity (Supplementary Figure S2A). We therefore compared the ability of the K12R mutant and K34R mutant forms of PEA3 to activate transcription in the presence of p300. While the K34R mutant showed virtually WT activity, the K12R mutant exhibited a substantial reduction in its trans-activation capacity (Figure 5B). Thus, the loss of acetylation and p300 binding in the K12R mutant correlates strongly with the loss of trans-activation capacity. Conversely, we also introduced glutamine residues in place of K96 and K222 in the K12Q mutant form of PEA3 to mimic acetylation. Mutation of K96 to glutamine caused a small increase in PEA3 activity (data not shown). However, the K12Q mutant form of PEA3 exhibited substantially elevated levels of transcriptional activity (Figure 5C), further underscoring the importance of acetylation at these two residues.

K96 was shown to be sufficient among the five lysines tested for binding to p300 (Figure 4), therefore we asked whether this residue might be sufficient for providing



**Figure 5.** The role of acetylated lysine residues in p300-mediated potentiation of PEA3 activity. (A–D) Reporter gene analysis using a luciferase reporter construct driven by five PEA3 (ETS) binding sites (shown schematically at the top). HEK293 cells were transfected with expression vectors for either WT or mutant PEA3 derivatives alone (grey bars) or co-transfected with p300 (black bars). Luciferase activities are the average of duplicate samples and are representative of two to three independent experiments. Activities are shown relative to the activity of the reporter alone (taken as 1). Western blots show the levels of PEA3 and p300 (A) and the levels of PEA3 in the absence (C) presence of co-transfected p300 (B and D) (dashed lines illustrate where two parts of a gel have been rejoined).

normal levels of PEA3-mediated trans-activation. Indeed, in the presence of p300, the K2345R mutant (which retains only K96) exhibited nearly WT activity whereas the K1234R mutant (which retains only K437) showed greatly reduced activity (Figure 5D; Supplementary Figure S2B).

These results demonstrate that at the functional level, multiple lysine residues contribute in a combinatorial manner to p300-mediated transcriptional activation through PEA3, and in particular point to a prominent role for K96, in combination with K222.

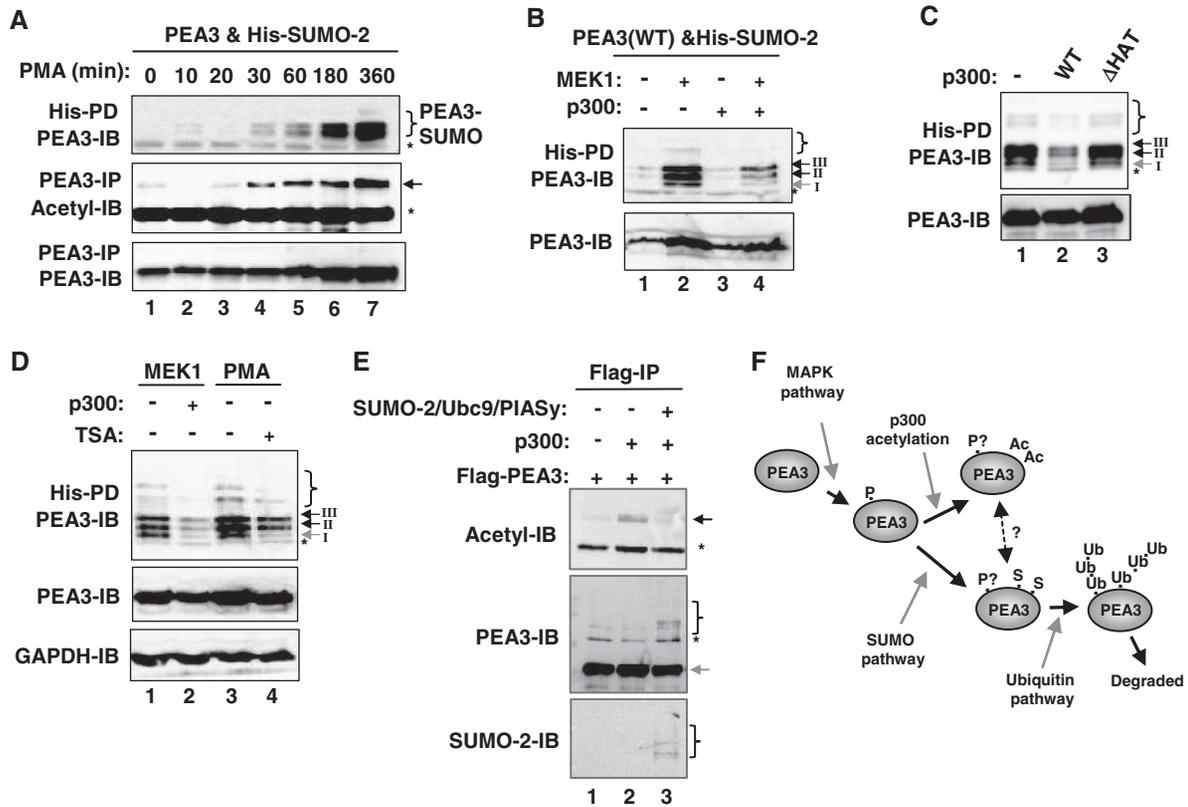
### The interplay between acetylation and sumoylation

The results so far clearly demonstrate an important role for lysine residues embedded within core sumoylation consensus motifs (Figure 3A) in p300-mediated acetylation events. As at least two of these sites, K96 and K256, are acetylated (Figure 3A) and sumoylated (14), this suggests that these modifications must be mutually exclusive events. To test whether this was the case, we first monitored the kinetics of PEA3 acetylation and sumoylation following the stimulation of cells with PMA. Significant increases in both acetylation and sumoylation of PEA3 could be detected 30 min after PMA stimulation (Figure 6A, middle and top panels, respectively). Acetylation and sumoylation then continued to rise over a 6-h period, most notably for sumoylation (Figure 6A; Supplementary Figure S3). Thus kinetically the two modifications initially follow similar time courses, although the relative levels of sumoylation appear to be greatly enhanced at later timepoints. Next, we overexpressed p300 and monitored PEA3 sumoylation levels. p300 caused a reduction in sumoylation levels as would be predicted if the same sites are being modified (Figure 6B and D). Moreover, the HAT activity of p300 is required for inhibiting sumoylation (Figure 6C), thereby ruling out a steric hindrance model where p300 binding would occlude access for the sumoylation machinery. Furthermore, we tested whether raising the acetylation levels in cells by using the HDAC inhibitor TSA would result in reduced sumoylation. Again, this mode of increasing acetylation resulted in a severe reduction in sumoylation levels, which was especially noticeable in the bottom sumoylation band which corresponds to sumoylation of K96 [Figure 6D; (14)]. Conversely, we raised the levels of PEA3 sumoylation by overexpressing components of the SUMO machinery and assessed the impact on PEA3 acetylation. As PEA3 sumoylation levels were increased, PEA3 acetylation levels were decreased (Figure 6E, lanes 2 and 3).

Together, these results demonstrate the mutually exclusive modification of PEA3 by sumoylation and acetylation and suggest that these modifications are dynamically regulated.

### DISCUSSION

Sumoylation has emerged as an important post-translational modification of transcription factors which can change their activities (reviewed in 13). However, it is becoming increasingly recognized that sumoylation is often a transient modification and can be dynamically regulated such as after the activation of intracellular signalling pathways (9). Part of this dynamism can potentially be achieved through competing modifications for the same lysine residue. This has been demonstrated to occur in several proteins such as HIC1 where antagonistic acetylation and sumoylation occurs on a lysine residue (20). Similarly, MEF2A is modified by SUMO and acetylation at the same residue, and in this case, dephosphorylation of MEF2A by calcineurin promotes a switch from sumoylation to acetylation (31). In the case of HIC1, the



**Figure 6.** Dynamic interactions between acetylation and sumoylation. (A) The kinetics of PEA3 acetylation and sumoylation were monitored in HEK293 cells co-transfected with expression vectors for PEA3 and His-tagged SUMO-2 and stimulated with PMA for the indicated times. Sumoylation was detected by pulldown with nickel-affinity beads (His-PD), followed by IB for conjugated PEA3 (top panel). Acetylation was detected following IP with anti-PEA3 antibody with IB with an anti-acetyl lysine antibody and input determined by IB with anti-PEA3 antibody. Bands corresponding to sumoylated and acetylated PEA3 are indicated by brackets and an arrow respectively. (B–D) PEA3 sumoylation assays were performed as in (A) but cells were co-transfected with expression vectors for (B) MEK1 and p300 where indicated, (C) WT p300 or p300 $\Delta$ HAT and treated with PMA for 6h, and (D) either a combination of MEK1 and p300, or treated with PMA and TSA. The locations of bands corresponding to sumoylation of PEA3 at K96 (I), K222 (II) or K256 (III) are shown by arrows and multi-sumoylation is shown by brackets. (E) HEK293 cells were co-transfected with the indicated combinations of expression vectors and PEA3 acetylation levels determined as in (A). Total PEA3 levels and PEA3 sumoylation levels in the IP were determined by IB with anti-PEA3 antibody (middle panel) and anti-SUMO-2 antibody (bottom panel). Bands corresponding to unmodified PEA3 (grey arrow), and acetylated (black arrow) or sumoylated (bracketed) PEA3 are indicated. Asterisks indicate bands arising from cross-reactivity with the antibody heavy chain. (F) Model depicting the sequential action of different pathways on PEA3. MAP kinase signalling initiates this cascade, partly due to phosphorylation (P) of PEA3, and promotes subsequent acetylation (Ac) and sumoylation (S), which in turn is important for polyubiquitination (Ub) and eventual turnover of PEA3. It is not known whether phosphorylation is retained in acetylated and sumoylated forms of PEA3, and this is indicated by a question mark. The double headed arrow and question mark denotes an alternative sequence of events in which acetylation and sumoylation are sequential events rather than mutually exclusive independent outcomes.

functional consequences of this switch have been established with switching from SUMO-dependent recruitment of the corepressor NuRD to loss of this corepressor upon acetylation (6). Here we show that the ETS-domain transcription factor PEA3 is subject to complex regulation by acetylation and sumoylation as it is also acetylated and sumoylated on the same lysine residues in a signal-dependent manner.

PEA3 is modified by acetylation and this is mediated by the acetyl transferase p300 (Figure 1). This effect appears specific as neither PCAF (Figure 1D) nor CBP (data not shown) appear to play major roles in PEA3 acetylation. Furthermore, PCAF does not appear to affect PEA3 function either as depletion of PCAF has little effect on PEA3-driven reporter gene activation (Figure 2). This contrasts with the related transcription factor ER81, where both p300 and PCAF appear to be involved (19).

Indeed, of the two sites identified in ER81 as important for acetylation, by p300 (K33 and K116), only K38 (equivalent to K33 in ER81) was identified in PEA3 by mass spectrometry as a p300-dependent acetylation site, and mutation of this site had no functional consequence (data not shown). The other lysine residue in ER81 (K116), equivalent to amino acid 123 in PEA3, does not appear to be conserved and is replaced by a glutamine residue in PEA3, which is often used as an acetylation-mimic and hence might mimic a constitutively acetylated state. Thus, the regulation of PEA3 by acetylation appears to be substantially different to ER81. Instead PEA3 acetylation takes place on numerous residues of which several overlap with the sites corresponding to lysines embedded in SUMO consensus motifs (Figure 3). Subsequent analysis demonstrated that amongst these sites, K96 appears to be one of the most

important for both acetylation and particularly for p300 binding, suggesting that it might act as a nucleating event for progressive PEA3 acetylation (Figure 4). It is therefore not clear what role acetylation at the other lysine residues plays in PEA3 function. However, the virtual complete loss of acetylation upon mutation of the five lysines embedded in SUMO consensus motifs (Figure 3C), suggests that the additional seven sites identified by mass spectrometry, which do not fit this category, are likely only present in sub-stoichiometric amounts. In addition to the sites identified by mass spectrometry, K222 also appears to be of particular importance in acetylation-dependent activities and functions alongside K96 (Figure 5). Thus while K96 in PEA3 is important and sufficient for many of the acetylation dependent activities, there appears to be a degree of functional redundancy with K222.

Lysine 96 is interesting as it is located within the consensus PDSM sumoylation motif ( $\psi$ KxEPxSP) which represents an extension of the core consensus motif. This motif is found in proteins such as MEF2A which are subject to control by phosphorylation (through proline-directed phosphorylation of the SP motif) and antagonistic modification by acetylation (31). The proline residue at position 5 appears important for the antagonism between sumoylation and acetylation (20). We previously demonstrated that the SP motif is important in promoting PEA3 sumoylation, thereby implicating proline-directed kinases in its regulation (14). Importantly though, activation of sustained ERK pathway signalling by PMA treatment activates both sumoylation [Figure 6; (14)] and acetylation (Figure 6) with similar kinetics. It is not yet clear whether this motif directs these events but it might be a contributory factor. Importantly though, as the induction of acetylation and sumoylation occurs with similar kinetics, this implies rapid dynamic interconversion of modification of distinct populations of molecules. By increasing the levels of acetylation or alternatively sumoylation, antagonistic effects are generated on overall PEA3 sumoylation and acetylation levels as might be expected from sharing the same modification sites (Figure 6). Thus it is clear that acetylation and sumoylation cannot co-exist on the same sites but as PEA3 contains three major acetylation sites, it is possible that molecules containing mixtures of acetylated and sumoylated residues might exist, and might represent transitions between fully acetylated and sumoylated states. Mechanistically, the antagonism seen by sumoylation of PEA3 might work similarly to sumoylation of PARP-1 which stops p300 from acetylating adjacent lysine residues (4), although this appears unlikely as the same residues are targets for both acetylation and sumoylation in PEA3. Importantly however, the occurrence of acetylation and sumoylation does not always imply functional antagonism as a similar interplay between acetylation and sumoylation was not evident with PGC-1 $\alpha$  (32).

Together, our results enable us to build a model for how different post-translational modifications impact on PEA3 activity (Figure 6F). First activation of the ERK MAP kinase pathway causes PEA3 phosphorylation (18) and leads to enhancement of acetylation and sumoylation. It

is not known whether phosphorylation is retained alongside sumoylation and/or acetylation. Both sumoylation and acetylation are associated with increased PEA3 activity. Thus, a sequential series of modifications by acetylation then sumoylation would be consistent with this common functional endpoint. It should however be noted that there are many possible intermediary scenarios due to the large number of acetylation and sumoylation events, so molecules with mixed populations of acetylation are likely (i.e. the model in Figure 6G is an over-simplification showing theoretical points where sumoylation would replace each of the acetylation events at the SUMO consensus motifs only). In one possible scenario, acetylation would either prime PEA3 for sumoylation, or alternatively would set a threshold whereby high levels of sumoylation would be needed to overcome the acetylation events. Such a scenario would occur on the lysine residues which are modified by either acetylation or sumoylation and would be consistent with the antagonism seen when manipulating either pathway (Figure 6). Conversely, it appears unlikely that sumoylation is required for acetylation as a PEA3 protein with mutations of the glutamic acid residues in all the core sumoylation motifs is still efficiently acetylated. However, this mutant does retain some sumoylation (unlike the equivalent lysine residue mutant) (14), and there is a reduction in acetylation levels, thus it remains possible that this basal level sumoylation might be sufficient to couple with acetylation events. This type of coupled regulation between acetylation and sumoylation has been proposed for p53 where sumoylation at K386 blocks subsequent acetylation by p300, but p300-acetylated p53 remains permissive for ensuing sumoylation at K386 (5). Sumoylation of PEA3 ultimately leads to the recruitment of RNF4 and its subsequent polyubiquitination and degradation (14). However, we cannot rule out that two parallel paths exist for PEA3 activation, one through acetylation and one through sumoylation. Indeed, mutating K96 and K222 to glutamine (i.e. mimicking acetylation) is sufficient to promote enhanced trans-activation by PEA3, which implies that subsequent sumoylation (at least not at these two sites) is not required for at least part of the acetylation-mediated increase in PEA3 activity. It is however clear that the regulatory events initiated by ERK MAP kinase signalling that impact on PEA3 function are complex and imply a large degree of dynamism between the various post-translational modifications involved.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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